

Production and characterization of reduced NAADP (nicotinic acid–adenine dinucleotide phosphate)

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The pyridine nucleotide NAADP (nicotinic acid–adenine dinucleotide phosphate) has been shown to act as a Ca^{2+} -releasing intracellular messenger in a wide variety of systems from invertebrates to mammals and has been implicated in a number of cellular processes. NAADP is structurally very similar to its precursor, the endogenous coenzyme NADP and while much is known about the reduced form of NADP, NADPH, it is not known whether NAADP can also exist in a reduced state. Here we report that NAADP can be reduced to NAADPH by endogenous cellular

enzymes and that NAADPH is functionally inert at the NAADP receptor. These data suggest that NAADPH could represent a mechanism for rapidly inactivating NAADP in cells.

Key words: calcium signalling, dehydrogenase, fertilization, reduced nicotinic acid–adenine dinucleotide phosphate (reduced NAADP), nicotinic acid–adenine dinucleotide phosphate (NAADP), sea urchin.

INTRODUCTION

Alongside InsP_3 and ryanodine receptors, it has been shown that a third intracellular channel, responsive to the pyridine nucleotide NAADP (nicotinic acid–adenine dinucleotide phosphate), can mediate calcium release from intracellular stores [1–3]. The initial reports came from sea urchin eggs, but NAADP has been shown to release calcium in an increasing number of systems, ranging from echinoderm eggs and plants to mammalian exo- and endo-crine cells [1–3].

The channel responsive to NAADP is pharmacologically distinct from those responsive to InsP_3 and cADP ribose and, unlike the other two, does not appear to mediate calcium-induced calcium release [1–3]. Furthermore, it has been shown by different techniques that the NAADP-sensitive Ca^{2+} pool is distinct from that sensitive to the other two mechanisms, i.e. the endoplasmic reticulum [4–6]. In sea urchin eggs, it has been recently postulated that this store might reside in the reserve granules, a lysosome-related organelle [7]. Alongside the NAADP receptor, recent reports suggest that NAADP might also act on the ryanodine receptor [8].

Recent research has been concentrated on the elucidation of potential roles of NAADP in cells. In mammalian cells, it seems that NAADP may act either as a trigger or a modulator of InsP_3 - and cADP ribose-mediated Ca^{2+} signals [9–11]. In echinoderm eggs, NAADP has been suggested to play a role in the characteristic Ca^{2+} transients seen at fertilization [12–14]. In sea urchins, it has been shown that NAADP is contained at high (micromolar) concentrations in the sperm cytosol and might be injected into the egg upon sperm fusion [13]. Furthermore, this finding was followed by a report that NAADP levels in sperm are increased following contact with egg jelly [14].

In addition to the Ca^{2+} transients seen at fertilization, large fluxes in both the levels and redox state of pyridine nucleotide coenzymes [NAD(P)] have been observed [15–17]. NAADP is closely related structurally to NADP, differing only in the substitution of a carboxyl group at the 3' position of the pyridine

for the amide. In the present manuscript, we have investigated whether NAADP can be reduced to NAADPH in a similar manner to the other endogenous pyridine nucleotides. This would be of particular interest since the NADP/NADPH ratio in cells generally favours the reduced form and it is possible that the same situation would apply for NAADP [18]. We now report that glucose-6-phosphate dehydrogenase, an enzyme capable of reducing NADP to NADPH, is also capable of reducing NAADP to NAADPH. Furthermore, NAADPH does not appear to have significant affinity for the NAADP-gated Ca^{2+} channel nor does it elicit Ca^{2+} release at what might be expected to be physiological concentrations. We therefore propose that reduced NAADPH could act as an inert pool of NAADP, providing a fast on/off signal for NAADP-induced Ca^{2+} release.

MATERIALS AND METHODS

Isolation and identification of NAADP-binding proteins

NAADP was immobilized via the pyridine ribose using an adaptation of the method described by Wilchek and Lamed [19]. Briefly, 10 μmol of NAADP were stirred, protected from light, for 1 h at room temperature in 1 ml of 100 mM sodium acetate/100 mM sodium periodate, pH 5.0. Excess periodate was precipitated by addition of 200 mM KCl. This was incubated for 5 min on ice before centrifugation at 20 000 g for 5 min. The supernatant was then stirred at 4 °C with 1 ml adipic acid dihydrazide-agarose beads in 100 mM sodium acetate, pH 5.0, for 3 h, protected from light, to allow coupling. The beads were washed twice in 1 M NaCl to stop the reaction, resuspended in PBS (50 % slurry) and stored at 4 °C.

Sea urchin egg cytosol was incubated with control or NAADP-agarose beads for 3 h at 4 °C in intracellular medium (Glu-IM; 250 mM potassium gluconate, 250 *N*-methylglucamine, 25 mM Hepes and 1 mM MgCl_2 , pH 7.2) in the presence or absence of 4 nM authentic NAADP. The beads were then washed five times in intracellular medium and proteins were then eluted by boiling in

Abbreviations used: NAADP, nicotinic acid–adenine dinucleotide phosphate; NAADPH, reduced NAADP.

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SDS loading buffer. Proteins were then separated by SDS/PAGE and visualized by Coomassie Blue staining. NAADP-binding proteins were identified by in-gel digestion of the protein bands with trypsin and identification of the resulting peptides by tandem MS [20]. Mass analysis was performed on a ThermoFinnigan LCQ Classic mass spectrometer using static nanospray infusion of the desalted digest mixture. Database searching was done with Mascot (<http://www.matrixscience.com>) and only statistically significant hits were considered.

Glucose-6-phosphate dehydrogenase assays

Enzyme assays were performed to test whether NAADP could be reduced to NAADPH by glucose-6-phosphate dehydrogenase using an adaptation of a protocol for NADP being converted to NADPH [21]. We incubated 10 units of the enzyme (glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*; Sigma) with 2 mM glucose 6-phosphate and 1 mM NAADP in a buffer containing 100 mM Tris/HCl, 500 μ M EDTA and 10 mM MgCl_2 , final volume 300 μ l. The reaction was allowed to proceed at 37 °C with the absorbance being measured on a spectrophotometer at λ_{340} .

Chemical synthesis of NAADPH

NAADPH was synthesized chemically using an adaptation of the method used by Ichinose et al. [22], substituting NAADP for NADP.

Purification and chemical characterization of NAADPH

Oxidized and reduced nucleotides were separated using DEAE-cellulose anion-exchange resin. Elution of nucleotides from this resin was performed with NH_4HCO_3 [22].

Briefly, the resin was washed in 30 mM NH_4HCO_3 and packed into a 20 cm \times 1 cm column and connected to a BioLogic Duo-Flow HPLC system (Bio-Rad). Nucleotides were loaded in 30 mM NH_4HCO_3 and were eluted with the following protocol (buffer A was double-distilled water and buffer B was 500 mM NH_4HCO_3): 0–8 min, 6 % buffer B; 8–15 min, linear increase of buffer B to 25 %; 15–39 min, linear increase to 60 % buffer B; 39–63 min, linear increase to 65 % buffer B; 63–75 min, linear increase to 75 % buffer B; 75–87 min, linear increase to 90 % buffer B; 87–99 min, linear increase to 100 % buffer B; 99–111 min, held at 100 % buffer B. Fractions were collected in a Bio-Rad 2128 fraction collector. Products were detected at A_{254} or A_{359} . Alternatively, products were separated on AG-MP1 resin (Bio-Rad) as described previously [26].

Malachite Green phosphate assay

Nucleotide concentrations were measured by estimating the 2'-phosphate content adapting the protocol of Lanzetta et al. [23]. In brief, 0.5 units of alkaline phosphatase were incubated with nucleotides in a buffer containing 20 mM Tris/HCl and 2.5 mM MgCl_2 , pH 10.5, at 37 °C overnight. Between 1 and 100 nmol of digested nucleotides were taken and incubated with 800 μ l of the assay buffer (0.045 % Malachite Green hydrochloride, 4.2 % ammonium molybdate in 4 M HCl and Triton X-100, 74.8:25:0.2, by vol.; incubated for 20 min before addition of nucleotides). After 2 min of incubation, 800 μ l of 34 % sodium citrate was added and the reaction left at room temperature for 40 min. The A_{660} of the samples was compared with that obtained from known concentrations of KH_2PO_4 .

Biological assays

Unfertilized sea urchin egg homogenates were prepared as described previously [4,24,25]. Fertilized sea urchin egg homogenates were prepared in a similar manner, except that after de-jellying the eggs, sperm was allowed to fertilize the eggs. The procedure was terminated by centrifugation at 13 000 g for 10 s, 5 min after more than 90 % of the eggs displayed a fertilization envelope. Ca^{2+} -release experiments were performed using an LS50B fluorimeter (Perkin-Elmer) using fluo-3 as the Ca^{2+} indicator [24,25]. Enzymically synthesized NAADPH was used for all biological assays. For pre-incubation experiments, 20 μ M NAADP or NAADPH (7.5 μ l) were incubated with an equal volume of 2.5 % homogenate for the indicated times (1 or 5 min). This reaction was then diluted 1:100 into 750 μ l of fluo-containing homogenate in the fluorimeter.

Radioligand-binding assays were performed using [^{32}P]NAADP as a tracer as described in [26]. To prepare radioactive [^{32}P]NAADPH, 100 nM [^{32}P]NAADP was used as a precursor.

RESULTS AND DISCUSSION

To identify NAADP-binding proteins in sea urchin egg cytosol, an affinity chromatography protocol was employed. A putative NAADP-binding protein was defined as (i) a protein that bound to NAADP-agarose beads but did not bind to control (adipic acid dihydrazide)-agarose beads and (ii) a protein that could be prevented from binding to NAADP-agarose by low concentrations (4 nM) of free NAADP. When bound proteins were stripped from the beads and analysed by SDS/PAGE, three specific bands were identified (Figure 1). MALDI-MS revealed that these bands corresponded to glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and isocitrate dehydrogenase. These proteins did not bind control adipic acid beads nor did they bind NAADP beads in the presence of competing NAADP (4 nM). Furthermore, when binding was allowed to occur, but the bound proteins were eluted with NAADP (4 nM) to increase the specificity of the assay, distinct bands could be observed with molecular masses identical to glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase.

To test whether NAADP could be reduced by dehydrogenases, we incubated glucose-6-phosphate dehydrogenase (10 units), together with 1 mM NAADP and 2 mM glucose 6-phosphate and employed the potential of reduced pyridine groups to absorb at λ_{340} . Upon addition of NAADP, an increase in A_{340} was observed (Figure 2A). This rise reached a plateau after 45 min, and did not occur in the absence of any one of the reagents, suggesting the reduction of NAADP to NAADPH. To analyse the products of this reaction, a sample was run on DEAE-cellulose anion-exchange resin. Products were detected at 254 and 359 nm and compared with the elution profiles of the original reactants (Figure 2B). The λ_{254} profile showed that authentic NAADP eluted at approx. 40 min (peak 1), while two new peaks, eluting at approx. 57 (peak 2) and 73 (peak 3) min, were observed after the reaction. When analysed at 359 nm, neither peak 1 nor peak 2 displayed absorbance. However, peak 3 absorbed moderately at λ_{359} . Since reduced pyridine nucleotides display a peak of absorbance at around λ_{340} , we then sampled the fractions on a spectrophotometer at λ_{254} and λ_{340} . The maximal λ_{340} absorbance detected co-eluted with peak 3. The λ_{340} absorbance of the peak was considerably lower than that at λ_{254} with the ratio A_{254}/A_{340} being 3.05 ± 0.016 . This compares with the measured A_{254}/A_{340} ratio for NADPH of 2.33 ± 0.037 . Retention times of peaks 1 (NAADP) and 3 mirror the relative retention times shown by NADP/NADPH

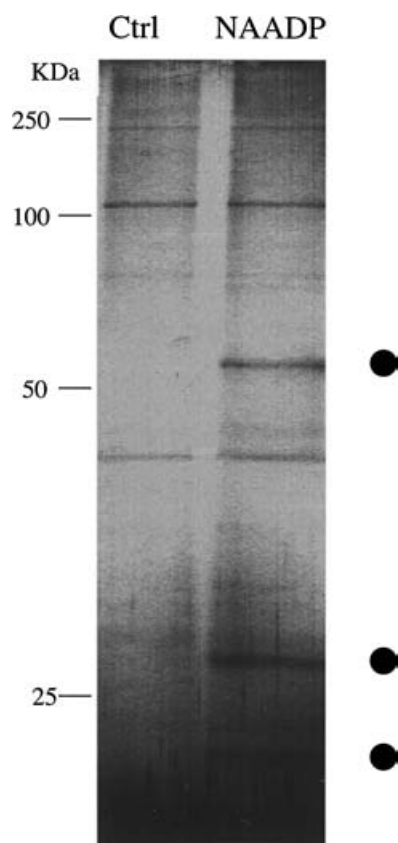


Figure 1 Binding of sea urchin cytosol proteins to control (Ctrl) or NAADP beads

Proteins were eluted with SDS loading buffer, run on a 5–12% acrylamide gradient gel and silver stained. Bands marked with black dots (glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, isocitrate dehydrogenase) represent specific NAADP-binding proteins and could also be eluted with 4 nM NAADP alone.

both with the protocol used here (results not shown) and as previously reported [22]. Further to this, a wavelength scan between λ_{240} and λ_{400} was performed on peak 3 and this was compared with the scans obtained from authentic NAADP and NADPH. All three compounds showed absorbance peaks at approx. λ_{260} (NAADP, 262 nm; NADPH, 262 nm; peak 3, 261 nm), while only NADPH and peak 3 showed absorbance peaks between λ_{300} and λ_{400} (NADPH, 344 nm; peak 3, 327 nm; Figure 3). Taken together, these data strongly suggest that the main product of the glucose-6-phosphate dehydrogenase reaction is reduced NAADP (NAADPH). To analyse whether glucose-6-phosphate dehydrogenase could convert smaller amounts of NAADP, 100 nM [32 P]NAADP was incubated under identical conditions to those described above and the products were separated by HPLC and quantified by scintillation counting. Under these conditions, $30.2 \pm 3.5\%$ of the original NAADP was converted in 45 min. Furthermore, when 100 nM NAADP was incubated in the presence of 100 μ M NADP, $44.6 \pm 3.5\%$ NAADP was converted. This suggests not only that NAADPH can be produced at the concentrations of NAADP that would be expected in cells (nanomolar) but also that endogenous NADP levels should not significantly interfere with this process.

The identity of the compound eluting in peak 2 is unknown but its absorbance at λ_{254} and its retention time on HPLC may suggest that it is a derivative of NAADP that retains both the adenine and the 2'-phosphate group. 2'-Phosphate-ADP-ribose has an

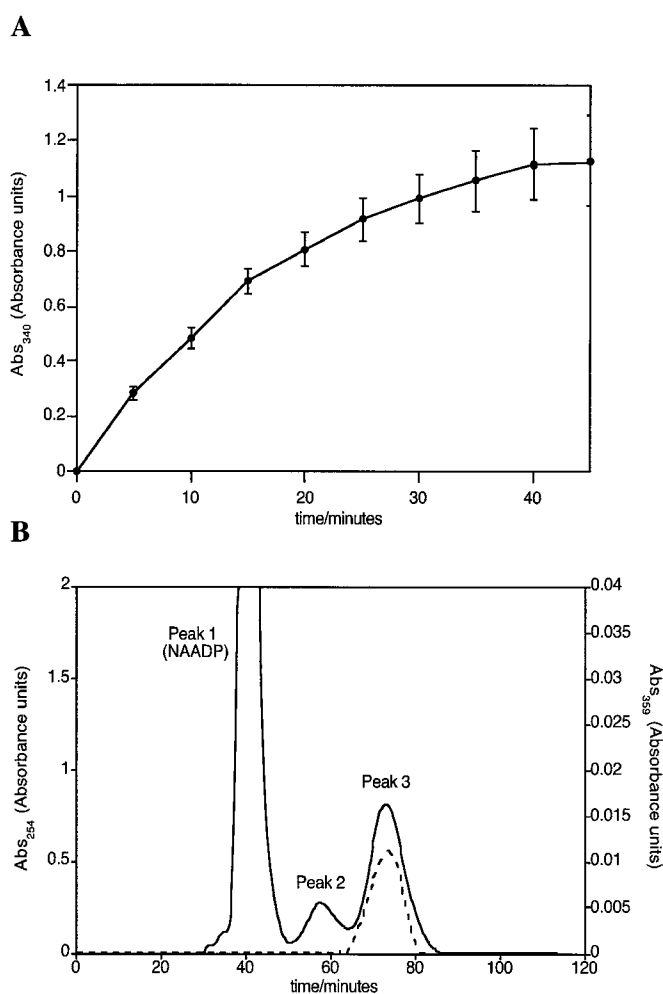


Figure 2 Reduction of NAADP by dehydrogenases

(A) Reduction of NAADP by glucose-6-phosphate dehydrogenase in the presence of glucose-6-phosphate. Production of NAADPH measured as the increase in A_{340} over time. Values are mean \pm S.E.M. from a representative experiment ($n = 5$). (B) Representative HPLC traces showing the product separation of the dehydrogenase reaction in (A). Nucleotides were detected at 254 nm (solid line) or at 359 nm (dashed line). Peak 1 corresponds to unreacted NAADP while peaks 2 and 3 correspond to the new products formed.

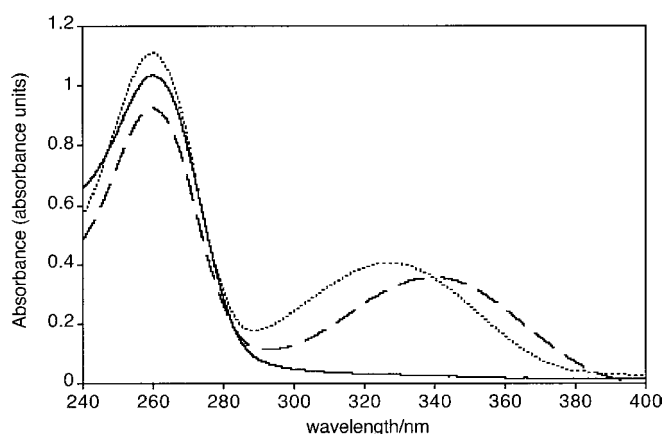


Figure 3 Wavelength scans performed between λ_{240} and λ_{400} for NAADP (solid line), NAADPH (dotted line) and NADPH (dashed line)

did not result in any loss of activity (Figure 5C). This supports previous reports that degradation of NAADP in unfertilized sea urchin egg homogenate is slow [29]. Conversely, incubating NAADPH with sea urchin egg homogenate resulted in a significant gain in activity (Figure 5D). Pre-incubated NAADPH cross-desensitized with NAADP (results not shown), and this would be consistent with a partial conversion of NAADPH to NAADP. If this were the case, an equilibrium assay such as [32 P]NAADP radioligand binding with NAADPH and NAADP should yield similar affinities and highlight a disparity between receptor affinity and Ca^{2+} release. Indeed, NAADP showed an affinity of 250 ± 19 pM ($n=9$) and NAADPH displayed a similar affinity (384 ± 50 pM; $n=9$). The similarity extended to the lack of co-operativity observed in either of the displacement curves, with Hill slopes close to unity (1.04 ± 0.07 and 0.90 ± 0.11 respectively). These data suggest that the high binding affinity and effect at high concentrations on Ca^{2+} release are both attributable to NAADP produced from 'inert' NAADPH. This is further strengthened by the observation that incubation of NAADPH with sea urchin egg homogenate produced a compound that co-eluted with authentic NAADP when analysed by HPLC (results not shown). To investigate whether NAADPH, as a low-affinity ligand, might have an antagonistic effect on NAADP-induced Ca^{2+} -release, 30 nM NAADP and 300 nM NAADPH were co-added to sea urchin egg homogenate (Figure 5E). The Ca^{2+} release observed upon co-addition of the two compounds was comparable with that observed upon addition of 30 nM NAADP alone. This demonstrates that the reduced compound is unable to antagonize the effect of the oxidized compound, strengthening the evidence that NAADPH lacks significant affinity for the NAADP-binding site.

The unfertilized sea urchin egg is unusual among cells since it is maintained largely in an oxidized state [15]. To support this, when either NADPH or NAADPH are incubated with homogenate, they are rapidly oxidized, as measured by their loss of λ_{340} absorbance (results not shown). This conversion to the oxidized form parallels the functional data for NAADPH shown above. It has been shown that at fertilization there is a shift from a largely oxidized to a largely reduced state [15]. To test whether this difference might affect the conversion of NAADPH to NAADP, homogenates were prepared from fertilized eggs. Incubation of NAADPH with fertilized homogenate produced a significantly lower amount of NAADP, as measured by the Ca^{2+} -release bioassay, compared with unfertilized homogenate (13.3 ± 11.0 versus 46.6 ± 6.8 relative fluorescence units; Figure 5F). This would suggest that NAADPH is more stable in the fertilized reduced environment.

In conclusion, we have demonstrated that the second messenger NAADP can be converted enzymically to its reduced form, NAADPH. By protein screening, we have shown that enzymes of relatively high abundance, such as glucose-6-phosphate dehydrogenase, are capable of such a conversion. Nonetheless, we cannot exclude the possibility that low-abundance NAADP-specific enzymes in the cell are responsible for regulated catalysis of this conversion or that the sea urchin egg enzymes detected may display a dual specificity for NADP/NAADP. At present, there are two possibilities for the role of NAADPH. First, NAADPH could be the precursor molecule of NAADP. If this were the case, cells could regulate Ca^{2+} release according to the redox state of the cell. For example, it has been shown that in mammalian cells extracellular messengers, such as angiotensin II [30], can activate an NAD(P)H oxidase, and this might lead to increases in NAADP from NAADPH. NADPH oxidase may also serve to increase NADP levels that can then be converted to NAADP. Secondly, since NAADPH is virtually inert towards the receptor, it could also

act as a degradative intermediate or temporary pool of inactivated NAADP to rapidly reduce second-messenger concentrations. This second mode could act in concert with the more extreme specific dephosphorylation pathway suggested as a degradative pathway in rat brain [31]. Consistent with the possibility that NAADP is reduced in sea urchin eggs is the observation that two of the enzymes found to show affinity for NAADP (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) undergo a massive up-regulation of activity following sperm–egg fusion [32]. This might serve, among other processes, to inactivate the NAADP introduced into the egg following sperm–egg fusion [13,14].

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